

L/TRDL-1, a Tumor Necrosis Factor-like Ligand, Stimulates Cell Death¹

γ Kelly,² Elizabeth Manos,² Gregory Jensen, Lincoln Nadauld, and David A. Jones³

² Cancer Institute, Division of Molecular Pharmacology, Salt Lake City, Utah 84112

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examined the activity of a new member of the tumor necrosis F family identified through Expressed Sequence Tag database using TNFα protein as the search query. We have termed this TNF-related death ligand-1α (TRDL-1α). Traditional cDNA screening identified two additional splice variants designated α and TRDL-1γ that differed from TRDL-1α by the deletion of two small regions within the protein coding region. TRDL-1α is identical in sequence to the recently described molecule, APRIL, that may induce cell proliferation. We found, however, that purified, FLAG-tagged TRDL-1α caused Jurkat cell death with kinetics that paralleled FasL. *In vitro* binding experiments demonstrated that TRDL-1α coprecipitated Fas and HVEM and suggested TRDL-1α as an alternate ligand for these receptors. TRDL-1 localized to chromosome 17p13.3 and its expression was widespread in normal tissues. Examination of 48 tumor samples revealed high levels of TRDL-1 expression in several tumors, including those from the gastrointestinal tract. Expression of TRDL-1 in COS-1 cells confirmed membrane association of TRDL-1, typical of TNF family members.

INTRODUCTION

TNF⁴ is the prototypic member of a family of cytokines with important roles in immune regulation, inflammation, and cancer (1, 2). The TNF family contains 14 members in addition to TNF-α including lymphotoxin α (3), lymphotoxin β (4), CD40L (5), CD30L (6), CD27L (7), OX40L (8), 4-1BB (9), FasL (10), TRAIL/APO-2 L (11, 12), TL-1 (13), TRANCE/RANKL (14, 15), LIGHT (16), TWEAK (17), and APRIL (18). These ligands are type II membrane-associated proteins (except lymphotoxin α) that share primary structure similarities that are confined to their COOH-terminal regions (19). This ligand group interacts with a growing family of target transmembrane receptors that are defined by cysteine-rich extracellular domains (2). In addition, several members of this receptor family, including TNF receptor 1, Fas, DR3, DR4, and DR5, contain a region of homology termed a “death domain,” which couples receptor activation to the apoptotic cellular machinery (20–26). The number of receptors contained within this family currently exceeds the number of putative ligands and suggests the existence of previously uncharacterized ligands. Identification of the cognate ligand for each receptor is necessary before a complete understanding of their roles in disease can be fully appreciated.

The importance of this cytokine family to immune system function is highlighted by phenotypic alterations associated with gene knockout experiments and endogenous mutations in mice. Mutations disrupting FasL or its counterpart receptor cause lymphadenopathy and

autoimmune disorders (27–31). In another example, CD40 mutations lead to hyperimmunoglobulin M phenotypes, suggesting an essential role for the CD40 pathway in B-cell affinity maturation and isotype switching (32). Inactivation of the TNF receptor type 1 pathway through knockout strategies generates mice that are highly sensitive to certain microbial infections, suggesting an important host defense role for TNF (33, 34). Lastly, inactivation of the murine lymphotoxin gene causes loss of peripheral lymph nodes (35).

Diverse roles for these cytokines are likely to extend beyond strict regulation of immune system function and into areas such as tumor development. A number of studies have demonstrated high levels of FasL expression in human tumors. Primary astrocytic brain tumors, colonic adenocarcinomas, and metastases of human colonic adenocarcinomas all show increased levels of FasL expression as compared with control tissues (36–38). Overexpression of these ligands could contribute to tumor development from two perspectives: (a) overexpression of death-inducing ligands, such as FasL, may provide a defense that protects tumor cells by killing intervening host immune cells (39–42); and (b) expression of TNF family ligands in tumors could create a chronic inflammatory condition that renders tumor cells resistant to immune destruction and confounds chemotherapeutic approaches that rely on tumor cell apoptosis. Evidence for this resistance can be seen in the observation that TNF fails to kill many types of cancer cells. Recent evidence suggests that TNF undermines its own killing powers by activating nuclear factor-κB, a key molecule that can block the apoptosis pathway (43, 44). Disruption of this protective mechanism may, therefore, sensitize cells to chemotherapeutic intervention.

Current interventional strategies targeting TNF pathways are challenged by a lack of understanding of how these pathways are regulated and dysregulated in disease. Furthermore, an incomplete roster of ligands and receptors adds to the complexity of selecting rational points for intervention. With this in mind, we have identified and examined the biological activity of a novel death-inducing ligand that is related to TNF and that we have termed TRDL-1. This ligand was identified previously as APRIL, a putative cell proliferation-inducing ligand (18). In contrast to this previous report, we found that APRIL/TRDL-1 stimulated Jurkat cell death and that APRIL/TRDL-1 binds to existing members of the TNF receptor family including, FAS and HVEM.

MATERIALS AND METHODS

Cell Culture Reagents. Jurkat and Cos-1 cells obtained from American Type Culture Collection were grown in RPMI 1640 and DMEM, respectively. All media were supplemented with 10% fetal bovine serum, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 50 units/ml penicillin G sodium, and 50 µg/ml streptomycin sulfate. Cells were grown at 37°C under 5% CO₂. All media and supplements were obtained from Life Technologies, Inc.

TRDL-1 Cloning and Sequence Determination. Clones corresponding to potential TNF-α matches were obtained from Genome Systems, Inc. IMAGE clone 727332 (TRDL-1α) was used to screen a human leukocyte cDNA library (Clontech Laboratories, Inc.). After an initial titration of the library, a total of 500,000 plaques were lifted in duplicate onto nylon filters. Phage DNA was denatured with the following wash protocol: 2× SSC for 1 min; 1.5 M NaCl, 0.5 M NaOH for 2 min; 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 2 min; and 2× SSC for 2 min. Filters were air dried and UV cross-linked using a Stratalinker (Stratagene). Filters were prehybridized in 6× SSC, 1× Denhardt's solution,

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The Huntsman Cancer Institute, Division of Molecular Pharmacology, Salt Lake City, Utah 84112

ABSTRACT

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100 µg/ml denatured salmon sperm DNA, and 0.5% SDS for 2.0 h at 65°C to block nonspecific DNA binding sites.

The probe was labeled with [α -³²P]dCTP (Amersham) to a specific activity of greater than 1×10^6 cpm/µg of DNA using an RTS RadPrime DNA labeling System (Life Technologies, Inc.). Unincorporated nucleotides were removed by passage over Chroma-Spin-30 (Clontech Laboratories, Inc.) size exclusion columns. Hybridization was performed in the same buffer as prehybridization for 12–14 h at 65°C with a probe concentration of 1×10^6 cpm/ml. After hybridization, unbound probe was removed by washing filters twice for 20 min each in 1× SSC, 0.1% SDS at room temperature, followed by two washes for 20 min each in 0.1× SSC, 0.1% SDS at 65°C. Filters were exposed to X-ray film for 12–14 h, with intensifying screens at –70°C. Plaques that showed duplicate hybridization signals were selected into an appropriate phage buffer and titered. Each positive plaque was then subjected to two additional rounds of hybridization screening to completely isolate the positive plaques from other species and to eliminate false positives. DNA was isolated, and the insert size was determined from plaques resulting from tertiary screens. Clones were sequenced by the Huntsman Cancer Institute Core DNA Sequencing Facility using ABI Prism BigDye Terminators and cycle sequencing with Taq FS DNA polymerase. DNA sequence was collected and analyzed on an ABI Prism 377 automated DNA sequencer (PE Applied Biosystems Division, Foster City, CA).

Northern and Dot Blot Analyses. Multiple human tissue mRNA blots I and II, a human cancer cell line blot, and a human RNA dot blot were purchased from Clontech Laboratories, Inc. A multiple tumor mRNA blot was obtained from Biochain Institute, Inc. The membranes were prehybridized in ExpressHyb (Clontech Laboratories, Inc.) for 2.5 h at 65°C and then hybridized with a random-prime, ³²P-labeled TRDL-1 probe for 2.5 h at 65°C. Unbound probe was removed by washing twice at room temperature in 2× SSC/0.05% SDS and twice at 50°C in 0.1× SSC/0.1% SDS. To quantify hybridization signals, blots were exposed to a PhosphorImager (Molecular Dynamics) screen for 6–24 h.

Expression of FLAG/TRDL-1 in Cos-1 Cells. Full-length TRDL-1 α was cloned into pFLAG-CMV-2 (Kodak) for expression in Cos-1 cells. Transient transfections were performed using Lipofectamine reagent (Life Technologies, Inc.), according to the manufacturer's protocol. Briefly, 10 µg of DNA were combined with 50 µl of Lipofectamine in 5.0 ml of serum-free Optimem (Life Technologies, Inc.). After DNA/Lipofectamine complex formation, the transfection mixture was added to 80% confluent Cos-1 cells in a 100-mm culture dish. After 4 h incubation under standard conditions, 5 ml of DMEM/10% fetal bovine serum were added, and cells were allowed to recover overnight. On the following day, the medium was removed and replaced with fresh, complete culture medium. TRDL-1 expression was monitored at ~48 h after transfection by analyzing cellular lysates for FLAG epitope.

Membrane Solubilization and Purification of FLAG/TRDL-1. Cos-1 cells transiently transfected with full-length TRDL-1 were incubated for 1 h at 4°C in hypotonic lysis buffer [50 mM Tris (pH 7.4), 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin]. Cell lysates were centrifuged at 500 × g for 7 min to remove the nuclei. The supernatant was then spun at 3000 × g for 15 min to pellet the membranes. Membrane fractions were washed twice with cold PBS and solubilized in a buffer consisting of 1% Triton-X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, and 10% glycerol overnight at 4°C. Particulate was removed by centrifugation at 12,000 × g for 10 min. Protein samples were loaded onto an anti-FLAG (M2) affinity resin (Kodak) at a flow rate of 0.2 ml/min. Columns were washed with 5 volume equivalents of PBS/0.1% Tween 20 and 5 volume equivalents of 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, and 10% glycerol. FLAG-TRDL-1 was eluted off the column by competition with 0.1 M FLAG peptide (Sigma). Fractions were assayed for the presence of TRDL-1 by immunoblotting. Fractions containing TRDL-1 were pooled and concentrated via a Centricon-10 (Amicon). Purity of the eluate was determined by SDS-PAGE and silver staining (Bio-Rad).

Generation of TRDL-1 Antibodies. A synthetic 15-amino acid peptide corresponding to residues 121–135 of TRDL-1 α was used to generate rabbit polyclonal antibodies. The peptide (CPINATSKDDSDVTE) was conjugated to keyhole limpet hemocyanin, and rabbits were immunized by Quality Controlled Biochemicals, Inc. Test bleeds were titrated by ELISA and tested by Western blot against a whole-cell lysate from Cos-1 cells expressing recom-

binant FLAG/TRDL-1. Sera showing good cross-reactivity were affinity purified to produce 5 mg of purified anti-TRDL-1 antibody.

Immunoblot Analysis. Samples were assayed for protein concentration using Bio-Rad protein assay kit. Protein samples (5–10 µg) were analyzed by SDS-PAGE on 10% tricine gels (Novex) and electrotransferred to PVDF membranes (Gelman Sciences). Blots were incubated for 1 h at room temperature in blocking buffer (5% powdered milk/PBS/0.1% Tween 20) and then with a monoclonal antibody to the FLAG epitope (Kodak) diluted 1:10,000 in blocking buffer for 1 h at room temp or overnight at 4°C. Blots were washed 3× in PBS/0.1% Tween and then probed with a secondary antibody conjugated to horseradish peroxidase. After washing 3× in PBS/0.1% Tween, FLAG protein was detected using a chemiluminescent substrate (DuPont NEN), according to manufacturer's instructions.

Receptor Binding Assays. All steps were carried out at 4°C. Sepharose beads coated with M2 antibody (anti-FLAG; Kodak) were blocked in 1% BSA for 1 h. After blocking, lysates (see above) from TRDL-1-transfected Cos-1 cells and untransfected cells were incubated with the blocked beads for 1 h. The beads were washed to remove detergent and unbound protein and then incubated with 250 ng of recombinant purified receptors fused to Fc (R&D Systems) for 2 h at 4°C in buffer containing 25 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM CaCl₂, and 1% BSA. After incubation, unbound proteins were removed by four consecutive washes with binding buffer lacking BSA. The remaining proteins were eluted by boiling in SDS-PAGE sample buffer and were loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes, and the blots were probed using an antibody specific for the Fc portion of the receptor fusions. Signals were quantified by scanning densitometry.

Cell Death Assays. Jurkat cells were resuspended in RPMI/0.5% FBS at 5 × 10⁴ cells/well in a 96-well culture dish and treated with vehicle alone, 50 ng/ml FasL (Upstate Biotechnologies, Inc.), 50 ng/ml TNF- α (R&D Systems), or 1.0 µg/ml purified TRDL-1. After 15 h of incubation at 37°C, cells were visualized by light microscopy and photographed. YO-PRO-1 dye (Molecular Probes) was then added at a final concentration of 1 µM. After an additional 3 h at 37°C, cells were analyzed on a fluorescence plate reader (Cytofluor II; Perceptive Biosystems) at excitation and emission wavelengths of 485 and 530 nm, respectively.

To determine a concentration curve, log dilutions of TRDL-1 ranging from 1000 to 0.1 ng/ml were added to cells as described above. After 48 h, cell numbers were quantified using a Coulter counter.

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1  MPASSPFL LAPKGPPGHMGCPVREPALSVALWLSWG TRDL-1 $\alpha$ 
1  MPASSPFL LAPKGPPGHMGCPVREPALSVALWLSWG TRDL-1 $\beta$ 
1  MPASSPFL LAPKGPPGHMGCPVREPALSVALWLSWG TRDL-1 $\gamma$ 

37 AALGAVACAHALLTQQTELQSLRREVSRLOQGTGGPS TRDL-1 $\alpha$ 
37 AALGAVACAHALLTQQTELQSLRREVSRLOQGTGGPS TRDL-1 $\beta$ 
37 AALGAVACAHALLTQQTELQSLRREVSRLOQGTGGPS TRDL-1 $\gamma$ 

73 QNGC GYP PWQSLPEQSSDALEAWENGERSRKRRRAVLT TRDL-1 $\alpha$ 
73 QNGC GYP PWQSLPEQSSDALEAWENGERSRKRRRAVLT TRDL-1 $\beta$ 
73 QNGC GYP PWQSLPEQSSDALEAWENGERSRKRRRAVLT TRDL-1 $\gamma$ 

109 QKQKKQHESVLHLVPINATSKDDSDVTTEVMWQPALRR TRDL-1 $\alpha$ 
109 QKQK[----]HESVLHLVPINATSKDDSDVTTEVMWQPALRR TRDL-1 $\beta$ 
109 QKQKKQHESVLHLVPINATSKDDSDVTTEVMWQPALRR TRDL-1 $\gamma$ 

145 GRGLQAAQCYGVRIQDAGVYLLTSQVLFQDVTFTHGQ TRDL-1 $\alpha$ 
145 GRGLQAAQCYGVRIQDAGVYLLTSQVLFQDVTFTHGQ TRDL-1 $\beta$ 
145 GRGLQAAQCYGVRIQDAGVYLLTSQVLFQDVTFTHGQ TRDL-1 $\gamma$ 

181 VVSREGQQGRQETLPRFCIRSMPSBPDRAYNSCYSAGV TRDL-1 $\alpha$ 
181 VVSREGQQGRQETLPRFCIRSMPSBPDRAYNSCYSAGV TRDL-1 $\beta$ 
181 VVSREGQQGRQETLPRFCIRSMPSBPDRAYNSCYSAGV TRDL-1 $\gamma$ 

217 FHLHQGDILSVIIIPRARAKLHLSPHGTFPLGFVKL TRDL-1 $\alpha$ 
217 FHLHQGDILSVIIIPRARAKLHLSPHGTFPLGFVKL TRDL-1 $\beta$ 
217 FHLHQGDILSVIIIPRARAKLHLSPHGTFPLC[---]L TRDL-1 $\gamma$ 

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Fig. 1. Primary amino acid sequences and alignment of TRDL-1 α , TRDL-1 β , and TRDL-1 γ . The predicted primary amino acid sequences of TRDL-1 α , TRDL-1 β , and TRDL-1 γ were aligned by the Clustal method. Areas where TRDL-1 α differs from TRDL-1 β and TRDL-1 γ are boxed.

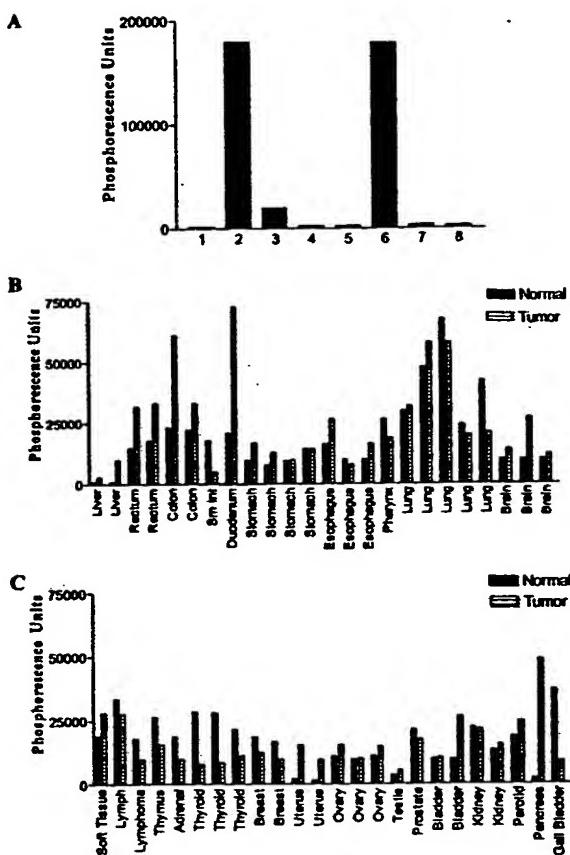


Fig. 2. Expression of TRDL-1 in cancer cell lines and human tumor specimens. *A*, expression of TRDL-1 was examined in eight human cancer cell lines (*column 1*, HL-60; *column 2*, HeLa S3; *column 3*, K-562; *column 4*, MOLT-4; *column 5*, Raji; *column 6*, SW480; *column 7*, A549; *column 8*, G361) by Northern analysis. Hybridization signals were quantitated using a PhosphoImager and are displayed in the plot as relative phosphorescence. *B* and *C*, a multiple human tumor blot containing 48 tumor and corresponding normal tissues was hybridized with a TRDL-1-specific cDNA probe. Hybridization signals were quantitated using a PhosphoImager, and a portion are displayed as relative phosphorescence.

RESULTS

Identification and Cloning of TRDL-1 α , TRDL-1 β , and TRDL-1 γ .⁵ To identify novel members of the TNF family of cytokines, we used the peptide sequence of human TNF- α and the BLAST (basic local alignment search tool) algorithm to query the dbEST database (45). Two sequences, AA405973 and AA477087, displayed interesting homology with TNF- α in regions where TNF- α is also similar to FasL, TRAIL, and other members of the TNF cytokine family (data not shown; Refs. 46 and 47). Ten additional expressed sequence tags with sequences that overlapped AA405973 and AA477087 defined a cluster that continued to share sequence similarity with the TNF family. Nucleotide sequence analysis of each clone confirmed our initial, computer-based alignments and strengthened the assignment of this cDNA into the TNF family. Of the clones examined, clone 727332 contained 1260 bp and a single, continuous open reading frame that predicted a 250-amino acid protein with a molecular mass of 27,432 daltons (Fig. 1). Primary alignment of the predicted open reading frame with TNF- α extended the critical homology regions, and the clone was renamed TRDL-1 to identify it as a new member of the TNF family.

Although the sequence of TRDL-1 encoded by clone 727332 was convincing enough to assign it to the TNF family, clone 727332 lacked a polyadenylation signal and poly(A)⁺ tail. This required additional cDNA cloning experiments to identify a full-length TRDL-1 cDNA. To isolate full-length TRDL-1 cDNAs, we screened a human leukocyte cDNA library using TRDL-1 as a probe. A total of 11 clones survived secondary and tertiary screening efforts with the TRDL-1 probe and were plaque purified. Restriction analysis of the 11 clones grouped them into two types of cDNAs (β and γ) that appeared to differ in organization from TRDL-1. Sequence determination of clones representing each of these variants confirmed their identity with TRDL-1 and revealed potential alternative splicing products (Fig. 1). TRDL-1 β was a 1684-bp clone that contained an open reading frame that predicted a protein of 234 amino acids and a molecular mass of 25,677 daltons. By comparison, TRDL-1 γ was composed of 1,607 bp and predicted a 257-amino acid protein with a molecular mass of 27,057 daltons. TRDL-1 β and TRDL-1 γ each contained a polyadenylation signal and a poly(A)⁺ tail. Fig. 1 shows the aligned, predicted amino acid sequences of TRDL-1 α , TRDL-1 β , and TRDL-1 γ . TRDL-1 β was identical to TRDL-1 α , with the exception of a 48-bp deletion that removed 16 amino acids corresponding to residues 113 through 128 of TRDL-1 α . TRDL-1 γ was also largely identical to TRDL-1 α but contained a 3' deletion of 181 bp that results in substitution of the four COOH-terminal residues of TRDL-1 α with a single leucine residue.

Tissue Distribution. We next examined the tissue distribution of TRDL-1 by performing Northern analyses on mRNA from various human tissues. Hybridization with TRDL-1 α identified mRNA species in most tissues. Highest levels of expression were seen in peripheral blood leukocytes, with intermediate levels of expression noted in pancreas, colon, small intestine, prostate, and ovary. There was little expression in skeletal muscle, thymus, or testis. Although most tissues expressed a 1.8-kb mRNA, peripheral blood leukocytes and lung expressed a message of 1.6 kb. Dot blot analysis of 34 additional tissues showed low levels of TRDL-1 expression in most tissues (data not shown).

To assess a potential role for TRDL-1 in tumor development, we next examined its expression in cancer cell lines (Fig. 2A). Strong expression of TRDL-1 (1.8-kb species) was observed in mRNA from HeLa and SW480 cells. Expression was undetectable in the other cancer cell lines examined and suggested the potential for cell type-specific regulation of TRDL-1 in cancers. We extended the cancer cell-based observations by surveying 48 human tumor biopsies compared with normal tissues for the expression of TRDL-1 (Fig. 2, B and C). A number of tumors showed higher levels of TRDL-1 expression as compared with adjacent normal tissues. Of note were gastrointestinal tumors, including rectum, duodenum, colon, stomach, and esophagus.

Subcellular Localization and Purification of TRDL-1 α . Secondary structure predictions for TRDL-1 suggested a single membrane-spanning region near the NH₂ terminus. To verify membrane localization, we next examined the subcellular localization of FLAG-tagged TRDL-1 α in COS-1 cells. To accomplish this, FLAG/TRDL-1 α was transiently expressed in COS-1 cells, and its distribution in cytosolic or membrane fractions was examined by Western analysis using antibodies specific for the FLAG epitope or TRDL-1. Fig. 3A shows the majority of FLAG and TRDL-1 cross reactivities in the membrane fractions of cells transfected with TRDL-1.

We then purified FLAG/TRDL-1 α from 1×10^8 COS-1 cells that had been transfected with pFLAG/TRDL-1 α . After a 96-h incubation, cell membranes were prepared by hypotonic lysis and differential centrifugation. Portions of membrane preparations were then combined with a solubilization buffer containing 10% glycerol and 1%

⁵ TRDL-1 α , TRDL-1 β , and TRDL-1 γ have the GenBank accession numbers AF114011, AF114012, and AF114013, respectively.

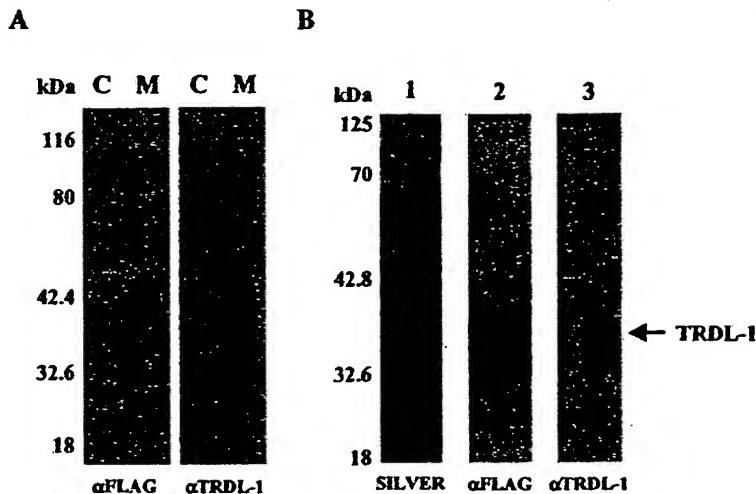


Fig. 3. Expression, subcellular localization, and purification of TRDL-1. *A*, COS-1 cells were transfected with pFLAG/TRDL-1 α (Lanes 1 and 2), and cells were harvested 48 h later. Membrane (M) and cytosolic (C) fractions were examined for FLAG/TRDL-1 expression using antibodies specific for FLAG epitope (right) or TRDL-1 (left). *B*, Cos-1 cells (1×10^6) were transfected with pFLAG/TRDL-1 and incubated for 96 h. After incubation, cell membranes were prepared by hypotonic lysis. FLAG/TRDL-1 was solubilized from the membrane in a buffer containing 10% glycerol and 1% Triton X-100. Solubilized TRDL-1 was adsorbed onto an anti-FLAG M2 affinity column and eluted using 0.1 M free FLAG peptide. Fractions containing TRDL-1 were identified by immunoblotting and combined. A portion was run on a 10% gel and silver stained to visualize TRDL-1 enrichment.

Triton X-100. Greater than 90% of the TRDL-1 protein was solubilized under these conditions. Solubilized TRDL-1 was then passed over the anti-FLAG M2 affinity gel to facilitate purification. The FLAG-tagged TRDL-1 protein was completely removed by the affinity matrix and was successfully eluted with 0.1 M FLAG peptide. Fig. 3*B* shows a silver-stained gel of purified FLAG/TRDL-1 and cross-reactivity of the purified protein with antibodies specific for the FLAG epitope and TRDL-1. We estimate a purification of FLAG/TRDL-1 at >80%.

Association with TNF Family Receptors *In Vitro*. Because TRDL-1 showed structural similarities to TNF family members, we reasoned that it may bind to TNF family receptors. We, therefore, examined the ability of TRDL-1 α to interact with purified TNF family receptors *in vitro*. This was performed by capturing FLAG/TRDL-1 α onto anti-FLAG affinity beads and combining these beads with purified TNF receptor 1/Fc, FAS/Fc, HVEM/Fc, TR1/Fc, TR2/Fc, and TR3/Fc fusion proteins. Precipitation of each receptor was assessed by immunoblotting for the Fc portion of the receptor fusions. Fig. 4*A* shows FLAG/TRDL-1 α -mediated precipitation of known TNF family receptors. Although there was detectable binding to all receptors above background, densitometric scanning revealed strongest binding of FLAG/TRDL-1 α to Fas (10.1-fold above background) and HVEM (11.2-fold above background) as compared with control beads and with the other receptors (Fig. 4*B*).

Induction of Jurkat Cell Death by FLAG/TRDL-1 α . Following the observation that FLAG/TRDL-1 α could bind to TNF family receptors *in vitro*, we next assessed whether it stimulated apoptosis in Jurkat cells. Fig. 5*A* shows the morphological consequences of exposing Jurkat cells to vehicle alone, 50 ng/ml FasL, 50 ng/ml TNF- α , or 1.0 μ g/ml TRDL-1 for 16 h. FasL and TRDL-1 each caused clumps of Jurkat cells to disperse, as compared with vehicle-treated cells or cells treated with TNF- α . The dispersal of Jurkat cells parallels cell death markers like caspase activation and annexin staining and suggests activation of similar pathways by TRDL-1 and FasL. Further inspection of the FasL and TRDL-1-treated cells showed typical apoptotic markers, including nuclear condensation, membrane blebbing, and cell shrinkage. In contrast, vehicle- and TNF-treated cells showed few of these characteristics at 16 h after treatment. In these same cells, viability was assessed by YO-PRO-1 dye uptake. Fig. 5*B* shows stimulation of YO-PRO-1 dye uptake in cells treated with FasL, TRDL-1 for 12 h but not in cells treated with vehicle and TNF- α . Finally, depletion of TRDL-1 by immunoprecipitation using the TRDL-1-specific antisera eliminated the death-inducing capability

and confirmed TRDL-1 as the active protein in the preparation (data not shown).

Finally, we established a concentration curve for TRDL-1-induced death by incubating Jurkat cells with 0–1000 ng/ml TRDL-1 for 48 h. After incubation, cell numbers were quantified by counting on a Coulter cell counter. Fig. 6*A* illustrates the concentration-dependent decline in cell number in response to TRDL-1. Replotting of this data revealed that TRDL-1-induced death is saturable (Fig. 6*B*). Maximal activity of TRDL-1 was seen at concentrations >100 ng/ml, with TRDL-1 showing an ED₅₀ of ~50 ng/ml.

DISCUSSION

We have identified a new member of the TNF family that we have termed TRDL-1. Two lines of evidence support functional similarities between TRDL-1 and the TNF family: (a) we found that purified, full-length TRDL-1 induced death in Jurkat cells with hallmark features of apoptosis. Killing of Jurkat cells by TRDL-1 was rapid, saturable, and occurred at concentrations typically seen with FasL and TNF- α . The rapid rate of death in response to TRDL-1 (within 12 h) is similar to death induced by FasL and suggests a potential interaction of TRDL-1 with Fas on Jurkat cells; and (b) we saw *in vitro* binding of TRDL-1 to several receptors of the TNF family. TRDL-1-coated beads precipitated purified Fas/Fc, TNF receptor 1/Fc, HVEM/Fc, TR1/Fc, and TR2/Fc fusion proteins. Of these, TRDL-1 preferentially bound Fas/Fc and HVEM/Fc. This suggests TRDL-1 as an alternate ligand for activating Fas. Although we saw binding of TRDL-1 to HVEM, HVEM lacks an intracellular death domain characteristic of apoptosis-inducing receptors (48). It is unlikely, therefore, that HVEM mediates the death-inducing effects of TRDL-1 in Jurkat cells. Future studies will be necessary to define the cognate receptor for TRDL-1 and to determine under what conditions TRDL-1 may activate Fas or HVEM.

While the manuscript was in preparation, Hahne *et al.* (18) reported the sequence of APRIL, a molecule that is identical in sequence to TRDL-1 α . Using a strategy similar to ours, these authors concluded that APRIL is a new member of the TNF family based on structural analyses. In contrast to our data, however, these authors reported that APRIL induced cell proliferation and that this proliferative signal could promote tumor cell growth. They propose that this activity is mediated through a novel receptor in that APRIL was incapable of binding purified TNF family receptors *in vitro*.

Although reconciliation of the differences between our observation

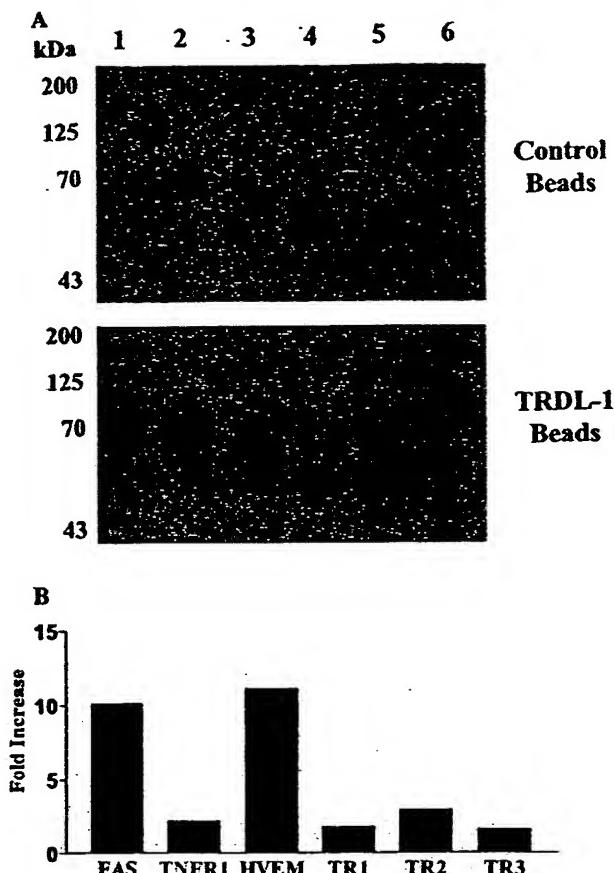


Fig. 4. *In vitro* binding of purified TRDL-1 to receptors from the TNF superfamily. *A*, control anti-FLAG M2 affinity beads (*upper panel*) or beads harboring ~25 ng of TRDL-1 (*lower panel*) were combined with 250 ng of each candidate receptor/Fc fusion protein including: FAS/Fc (*Lane 1*), TNF receptor 1/Fc (*Lane 2*), HVEM/Fc (*Lane 3*), TR1/Fc (*Lane 4*), TR2/Fc (*Lane 5*), or TR3/Fc (*Lane 6*). Binding reactions were incubated at 4°C for 2 h, and unbound proteins were removed by four consecutive washes with binding buffer. Remaining proteins were eluted by boiling in SDS-PAGE sample buffer and loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes, and the blots were probed using an antibody specific for the Fc portion of the receptor fusions. *B*, signals in *A* were quantified by scanning densitometry and TRDL-1-specific binding presented relative to binding in control beads.

and those of Hahne *et al.* (18) will require additional experimentation, differences in strategies for production of bioactive APRIL/TRDL-1 protein may account for the discordant conclusions. APRIL was produced as a soluble protein that lacked 110 amino acids from its NH₂ terminus (18). After our initial identification of TRDL-1, we expressed and purified a soluble construct that lacked 53 amino acids from its NH₂ terminus. This truncation removed the membrane-spanning region and allowed for more convenient purification. Addition of truncated TRDL-1, however, to a number of cell lines including Jurkat cells failed to elicit apoptotic responses, even at concentrations of TRDL-1 as high as 1.0 µg/ml (data not shown). We, therefore, turned to production of full-length TRDL-1 and witnessed the appearance of death-inducing activity as reported herein.

The observed lack of activity of truncated TRDL-1 could result from the inability of these molecules to form homotrimers. Trimerization of TNF family members could be central to the ability of the molecules to efficiently bind to and activate target receptors. Zhang *et al.* (49) have shown that residues near the membrane-spanning helix of TNF-α are critical to trimerization of these molecules and biological activity. Further, Schneider *et al.* (50) demonstrated that the

apoptotic activity of soluble FasL was reduced 1000-fold as compared with the membrane bound form of FasL. However, soluble FasL retained its ability to interact with Fas, and restoration of its cytotoxic activity was achieved, both *in vitro* and *in vivo*, with the addition of cross-linking antibodies (50). This suggests that the truncated form of FasL is not able to form trimers. Because we see both structural and functional similarities between TRDL-1 and FasL, it is possible that deletion of a significant portion of the APRIL/TRDL-1 NH₂ terminus could alter its activity. It is also possible that truncation of APRIL/TRDL-1 could alter receptor binding specificity and, therefore, elicit different responses. It will be critical to the final assignment of APRIL/TRDL-1 function to determine the trimerization state and receptor binding capabilities of the full-length molecule compared with various NH₂-terminal truncations.

TRDL-1 expression was fairly widespread in normal tissues and is similar to that reported previously for TNF, TRAIL, and TWEAK (11, 17). We saw the highest levels of expression in peripheral blood leukocytes that also displayed a message size that was unique as compared with other normal tissues. This unique message size could arise from alternate splicing of TRDL-1 mRNA. The possibility of alternate splicing was confirmed by our identification of TRDL-1 β and TRDL-1 γ . These two cDNAs were obtained by screening a

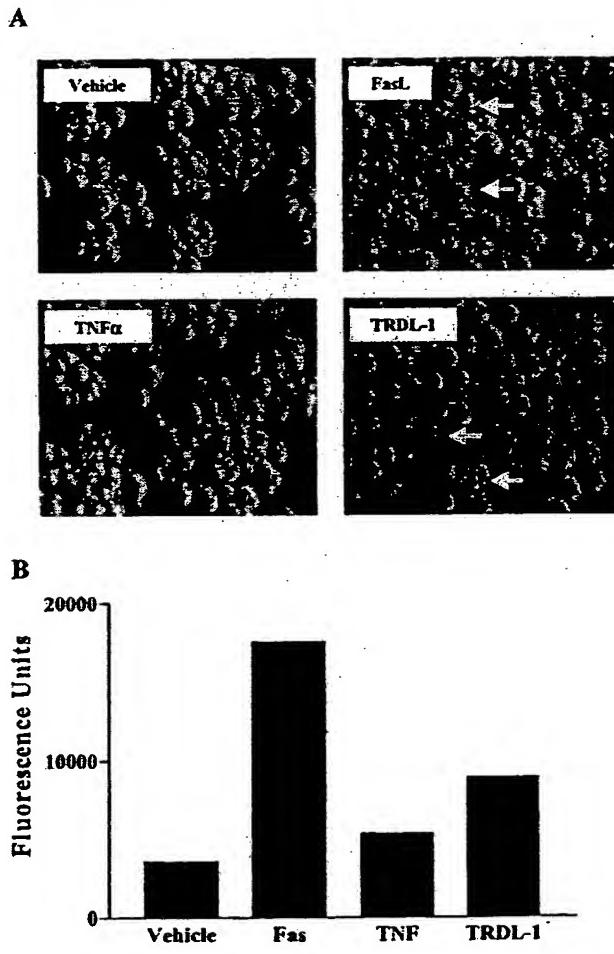


Fig. 5. TRDL-1 induction of Jurkat cell apoptosis. Cultures of Jurkat cells (5×10^4) were incubated with vehicle, 50 ng/ml FasL, 50 ng/ml TNF, or 1.0 µg/ml TRDL-1. After incubation, cell death was assessed by visual inspection (*A*) and by monitoring the uptake of YO-PRO-1 dye (*B*). Dye uptake was quantified using a fluorescence plate reader, and data are presented as arbitrary fluorescence units.

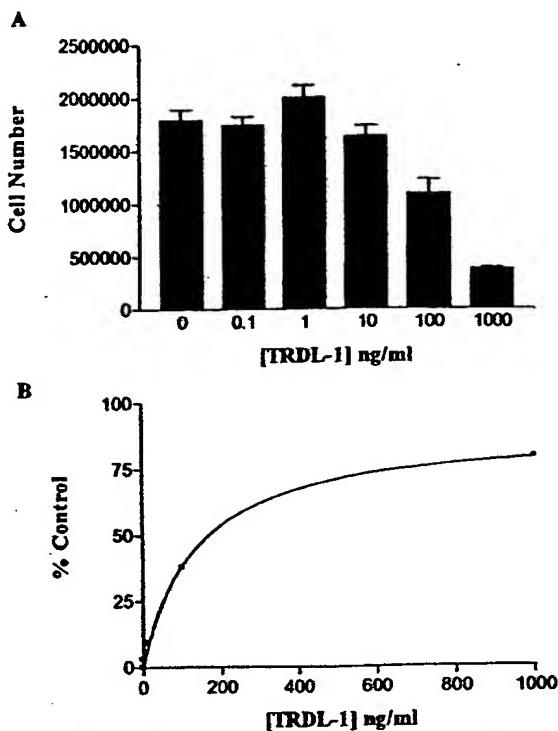


Fig. 6. Saturable killing of Jurkat cells by TRDL-1. A concentration curve for TRDL-1 α killing was performed by incubating triplicate cultures of Jurkat cells (5×10^6) with 0–1000 ng/ml FLAG/TRDL-1 α for 48 h. After incubation, cell number was determined by counting with a Coulter counter. A, cell counts are presented as means (bars, SD) and B, as a percentage of maximum death to show saturation.

leukocyte cDNA library with TRDL-1 α , TRDL-1 β and TRDL-1 γ each contained specific deletions as compared with TRDL-1 α that would result in translation of TRDL-1 α , TRDL-1 β , and TRDL-1 γ messages into three distinct proteins. The size differences in mRNAs we saw by Northern analysis correspond closely with size differences between TRDL-1 α /TRDL-1 β and TRDL-1 γ cDNAs. Assessment of the functional consequences of alternative splicing warrants further investigation.

We saw differential expression of TRDL-1 in tumor tissues that suggests a potential role for TRDL-1 in tumor development and maintenance. Our survey of 48 different tumors compared with adjacent normal tissues showed increased expression of TRDL-1 in a number of tumors, particularly those derived from the gastrointestinal tract. Tumors from rectum, duodenum, colon, stomach, and esophagus showed higher levels of TRDL-1 expression than those seen in adjacent normal tissues. This increased expression in tumors is similar to that seen for expression of FasL. Elevated levels of FasL have been observed in a variety of tumors, including those from the gastrointestinal tract (37, 38). The role of FasL in these tumors is unclear but it may provide a tumor defense against the host immune system or stimulate a chronic inflammatory condition (39–41, 43, 44). The role for TRDL-1 in tumor development is uncertain but may parallel those proposed for FasL.

In summary, we are presenting data supporting a role for APRIL/TRDL-1 as a death-inducing ligand of the TNF family. The ability of full-length APRIL/TRDL-1 to kill Jurkat cells and its overexpression in human tumor samples may offer a molecular mechanism for tumor cells to evade elimination by host immune cells. The potential for TRDL-1 to contribute to tumor development warrants further study that will focus on demonstrating TRDL-1 activity in tumor specimens.

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